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ance Notes on Codes and Abbreviations" appearing at the begin-
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(54) Title: CRYOPRESERVED AMNIOTIC HUMAN CELLS FOR FUTURE THERAPEUTIC, DIAGNOSTIC, GENETIC AND OTHERS USES

(57) Abstract: This invention belongs to the field of biotechnology, cryobiology and human therapeutics. Object of the invention are a) amniotic cells originating from amniotic fluid that surrounds the embryo in its early development stages, b) amniotic cells that at early embryonic stage are floating within the human embryo-clone, c) every biological material which will be created directly or indirectly from the above amniotic cells, as well as every biological product or by-product, which will be the outcome of the use of the said cryopreserved amniotic cells, through their reproduction or multiplication under the same or any other modified form and which will possess the same genetic properties and applications. These amniotic cells are isolated from their natural environment and are care preserved in deep-freezing for very long periods of time after their own natural life-span, which is short within their natural environment, that is within the amniotic fluid and within the embryo-clone. The purpose is the genetic and medical use of these cells at a time posterior to their natural destruction. In this way, we succeed to use primal, undifferentiated and genetically identical cells of each human being for diagnosis and therapy of genetic or non-genetic diseases, malfunctions and accidents, where these are known nowadays or shall be applied in the future.

WO 01/23532 A1



TITLE : Cryopreserved amniotic human cells for future therapeutic, diagnostic, genetic and others uses.

DESCRIPTION

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Technical field of the invention

The invention refers to the field of biotechnology and more particularly to the field of cryobiology. The invention applies to genetic diagnosis and through this, to genetic therapies of human diseases or life theater accidents by use of already cryopreserved human amniotic cells.

State of the art

Within the amniotic fluid which surrounds the embryo there exist amniotic cells. These cells are biological elements (material) that exist outside of the body and of the development of the embryo. These amniotic cells originate from embryonic cells which are driven away from the embryo during pregnancy, since the early embryonic stages and are moving freely inside the amniotic fluid that surrounds the embryo, where they remain during pregnancy.

The amniotic cells are non-differentiated (primal) cells and are genetically identical with all other cells, differentiated or not, of the embryonic body from which they come from. The amniotic cells can easily be grown and multiplied in cell cultures.

Until now, the amniotic cells are lost in the environment together with the amniotic fluids at birth and they cannot be used for the embryo's benefit in its future life, which means after birth. Therefore, all humans loose at birth a unique and indispensable genetic material forever.

There is no way, under natural conditions, for the amniotic cells to be preserved in a living condition after birth and after the loss of amniotic fluid.

Nowadays, the only known use of amniotic cells is taking place before birth, during pregnancy and is taking place for clearly prenatal testing via amniocentesis.

More specifically, amniotic cells are taken together with amniotic fluid
5 during pregnancy via amniocentesis for the diagnosis of biochemical, cellular, and chromosomal abnormalities of the embryo. During this procedure (Modern Genetics, F.J. Ayala & J.A. Kiger Jr. ed. Benjamin, Cummings, p.p. 722-724), a sample of 10-15ml amniotic fluid that surrounds the embryo is taken by using a surgical syringe between the
10 14 and 16th week of pregnancy. Amniotic cells may also be taken during earlier stages of pregnancy by other means.

Then, the amniotic cells that exist in the amniotic fluid are separated by centrifugation and on them chromosomal number and aberrations are viewed. Besides, other biochemical tests are also performed on the
15 amniotic fluid to see if some known genetic diseases out of the approximately 5000 existing ones are genetically determined in the embryo. Upon the results of this test depends the embryos' life, i.e. the interruption or not of pregnancy.

In the case that the amniotic sample is lost or in cases where more or
20 new material is needed, it is required that a second amniocentesis takes place, if this is allowed by the pregnancy stage.

Object of the invention

The object of the present invention are the amniotic cells, that is cells
25 which exist in the amniotic fluid surrounding the embryo. According to the present invention these cells are isolated from their natural environment and preserved in deep-freezing in a number of samples. Their life span is thus extended longer than their natural life-cycle, for long periods of time, with the goal to multiply and use them in the future for diagnostic,
30 therapeutic and other purposes for the after birth life of the embryo.

Object of the invention are amniotic cells which have been multiplied before their storage, or which have been multiplied after their storage, their thawing and their new cryo-preservation.

It is known that the human body in the different stages of its formation (i.e. embryonic development) and its development cannot be the object of an invention for which a patent may be granted. However, one
5 element (cell) which is isolated from the human body, such as the amniotic cells in the case of the present invention that are isolated by themselves and are flowing freely into the amniotic liquid, with the goal of being destroyed at birth, may represent a patentable invention. This is so, even if the genetic make-up of said the element (the amniotic cell
10 which, according to the invention, has been preserved in a viable-useful state longer than their natural life span) is the same with that of the natural element, that is with the amniotic cell that is part of the amniotic fluid at the time of birth.

15 Technical problem

The existing technical problem, that is solved by this invention is that nowadays, amniotic cells are not preserved in a viable - useful condition after birth, so that they be used in the after-birth life of the embryo and after the amniotic cells' natural destruction.

20

The amniotic cells that are coming from the amniotic fluid have, in comparison to other cells, the following advantage and uniqueness: they are cells that are primary, primal and less differentiated to a great extent, as well as genetically identical to all other cells (differentiated or not) of
25 the embryo's body, from which they originate. Due to these properties the amniotic cells can be the basic material for the production by differentiation of nearly any future differentiated cell of the body, which (differentiated cell) they can substitute genetically, functionally and physiologically. It is thus possible that epidermal cells are produced from
30 amniotic cells for use in plastic surgery.

Under certain conditions, amniotic cells have the prerequisites and the possibility to be differentiated into a number of the 200 existing different human body cells' categories.

Amniotic cells cannot be developed and differentiated by themselves; they have the potential though to be differentiated under specific control lab conditions that are constantly expanding and improving, into categories of differentiated cells that may be of future use to the embryo, that is in its after-birth life.

In the existing technical state of the art, it is not possible for diagnosis of new genetically determined diseases to take place on amniotic cells, besides those that are already known today, due to the non preservation of amniotic cells after birth. For genetic diseases that are already known to science, the only possible diagnosis on amniotic cells is prenatal diagnosis that takes place before birth. However, for diseases that will be known to science in the future, at a stage after the embryo's birth and at any age, it is no longer possible to use amniotic cells for diagnostic purposes in relation to those, because today these cells are lost during birth.

Besides, with the existing technical state of the art, it is not possible to appreciate the genetic predisposition for the development of diseases such as breast cancer, prostate cancer, nor is it possible to appreciate other genetically related genetic diseases like hypertension and heart diseases by using amniotic cells. The genetic cause of these diseases is still unknown, but this might be known in the future, after birth and during life of the human being, whose amniotic cells have been preserved according to the present invention. Thus, for the human being, whose amniotic cells have been preserved after birth, it will be possible at least to diagnose harmlessly genetic diseases.

Amniotic cells are unique cell material that cannot be replaced, which may serve not only for diagnostic purposes, but can also provide the basic cell material for gene and genetic therapy. Under the current state of the art, it is not possible to use amniotic cells for diagnosis or treatment of diseases, the treatment of which is still unknown during birth, or of day life accidents that may take place after birth, as well as of infectious diseases after birth. The reason for this is that following birth,

there do not exist any amniotic cells preserved somewhere for use after birth.

According to the present invention, amniotic cells that are preserved in a viable state after birth offer the possibility to diagnose new genetic diseases and to appreciate the genetic predisposal for the development of diseases, as well as to apply methods of gene or other genetic treatment in the case of genetic relative diseases, accidents and infectious diseases, during each person's life.

Amniotic cells according to the present invention can also be used to produce cells resistant to pathogens, viruses, bacteria, to be used for the creation of new cell lines, for the treatment of wounds, burns, for the addition of tissues for therapeutic or aesthetic reasons.

Another application of the present invention is that it offers the possibility to create healthy cells, tissue and organs from the amniotic cells that are stored and preserved in a viable state. These cells, tissue and organs can be used to replace non-healthy or malfunctioning similar ones and they can also be used to produce genetically identical copies - clones of the person they belong to.

These above mentioned new products, which may have been genetically modified or not, are genetically identical to corresponding cells, tissue and organs of their owner who is the recipient. Therefore, these are compatible with him, which means that when drafted, they have extremely few possibilities to be rejected. For instance, such cells are the muscular cells of cardiac valves, among others.

Due to their preservation according to the present invention, the stored amniotic cells even if contaminated by infection factors such as HIV virus, do have the possibility to be freed by the infection factors by means of specific culture condition. Therefore, they can be used as healthy amniotic cells for therapeutic and other uses thereafter.

Another application of the present invention is that due to the preservation of viable amniotic cells, we may establish a person's

genetic identity (f.e. DNA fingerprint) which may follow the person during his whole future life. The genetic identity may be established when the sample is being taken, or later, after thawing of the preserved cells. Following the existing state of the art, in cases where it is necessary to
5 verify the genetic data of an infant in cases of erroneous information, unwanted adoption or unwanted paternity recognition, as well as of paternity matters for succession, it is necessary to take blood samples from both the father and the child for testing. Because of the present invention, such a new test will not be necessary because each human
10 being may have a ready genetic identity and stored genetic material. The same application is possible also when it is not possible to take a genetic sample in the case of a deceased person, which is necessary for criminology.

Establishing the genetic identity of persons with the use of amniotic cells
15 preserved according to the present invention offers the unique advantage of the creation of collective ready genetic data. This collective data is providing also the potential of unique genetic properties to be discovered and consequently to be used and exploited for research, diagnostic, therapeutic and genetic uses.

20 For example, while a human being was initially a HIV carrier, at a further stage of his life he is becoming virus-free and it is discovered that this is related to the possession of a specific genetic factor, which has been discovered to be the reason for the discard of the virus. By use of the collective genetic data assembled from the establishing of genetic
25 identities it is possible to find out who else possesses the same genetic factor and this in turn may be used for the above purposes.

Another application of the present invention is that amniotic cells that are preserved according to the present invention for uses that will take place after birth, uses in respect to the donor or his next of kin, do own and
30 preserve certain qualities due to the present invention. More specifically, they can be used to create differentiated human cells with much larger probability of success than any other cell category, because they are undifferentiated and genetically related to the recipients.

The stem cells, which are used today with the existing state of the art after being cryopreserved, are coming from the umbilical cord or from bones' marrow tissue. These cells have no relation to the amniotic fluid and what this includes, they are cells more differentiated in comparison to amniotic cells, and due to this differentiation it is relatively more difficult that they be differentiated in other cell categories. Besides, these stem cells also belong to tissues of the embryo, i.e. they are part to the development of the embryo, they differ morphologically and physiologically from amniotic cells and they are used in particular for cancer therapy.

Amniotic cells which are preserved viable and useful for future uses at a time after human birth and which are the object of the present invention, possess due to the present invention certain properties: because they are primal and undifferentiated, they can be used to produce differentiated cell lines of the human body with much greater probabilities of success comparatively to other existing cell categories; they can also be used for detection and therapy of diseases that appear after human birth, i.e. cancer, for genetic engineering and therapy and for curing infectious diseases and accidents that may happen during human life.

One way of taking amniotic cells and separating them from the amniotic fluid for their preservation according to this invention is the following:

During pregnancy, a quantity of amniotic fluid is taken with the amniocentesis process, or in any other way.

1. The amount of amniotic fluid varies between 20 to 80 ml for the needs of the present methodology.
2. The amniotic fluid is properly centrifuged and the amniotic cells are taken from the pellet.
3. The original amniotic cells are cultured by using one of the existing cultures substrates, such as MEM medium.

4. Cryoprotective compounds such as glycerine, DMSO or polyethylene glucols in concentrations between 5 to 50% are added to the amniotic cells.
5. A line of five vial from the start culture is produced and five more lines with 5 vials each, from the rest of the culture, are created by seeding. Thus, we have the first line, which consists of 5 vials on which we are working performing the necessary tests and five more lines, each with five vials, for the deep-freezing process.
6. The temperature of the samples (vials) starts falling via a very sensitive computer controlled system at a pace not more than one degree Celsius (1°C) per min.
7. Ice nucleation process starts by dropping microcrystals of ice so as to create ice from outside the cells, and so as to avoid the creation of crystals within the cells, which will destroy them.
8. Finally the temperature starts dropping faster up to the 150 degrees Celsius below zero (-150°C) and usually the vials are kept in liquid nitrogen (-196°C) where the total of the 25 vials are kept, see original paper of Pentz and Horler. (Pentz S. and Horler H. J. Med. Genet. 1980, Dec. 17 (6) : 472-5).
9. There is a number of thawing processes that are known and that can be applied. In general, the thawing process is nearly a reverse course of the freezing process, where the main consideration is again to avoid ice crystal formation within the cells and the osmotical removal of the cryoprotective compounds of glycerine or DMSO. It has recently been mentioned that even simple exposure to environmental temperature may be equally useful.

The amniotic cells can be prepared for preservation also in other ways, for example using freezing, dehydrating and closing the amniotic cells in polymers or liposomes etc., and then deep-freezing storage takes place as above or at much higher temperatures than that of liquid nitrogen (-196°C).

The amniotic cells' mixture that is being cryopreserved as described above can differ as to the degree of amniotic cells' concentration: from nearly only amniotic cells, up to pure amniotic fluid.

5 Freezing processes similar to the above described, are used in early human embryos' deep-freezing during the process of artificial insemination. These embryos are much more sensitive to cryopreservation than amniotic cells, because as they are differentiated and developed they have larger probabilities of being destroyed.

10

In the near future, new, better, simpler and less expensive methods of cryopreservation of amniotic cell will apply.

The present invention provides the possibility to preserve amniotic cells
15 of human embryos for a long time after birth.

One further object of the present invention is the amniotic cells that can be produced during the life-span of a human being, in other words after birth. These amniotic cells can be produced today using adults'
20 mammary gland cells or even epidermal cells.

The experiments at Roslin Institute in Scotland have proven that with the nuclear transfer technology from a mammary gland cell to a sheep ovary from where the nucleus had been removed, it is possible to create a viable embryo-clone, Dolly. With an approximately similar technology
25 human embryo-clones may also be produced.

It is permitted and legitimately and scientifically acceptable nowadays to produce human clone embryos and to store them up to 14 days of age. It is probable that this time limit will be extended in the future to longer than 14 days, for experimental and medical, genetic.

30

After a human embryo is created in the above way, the said embryos contain inside them in their early stages freely-floating amniotic cells. These early amniotic cells are ignored today for the existing level of state

of the art, that is they are lost after the termination of life of the embryo-clone. There is no way to preserve these amniotic cells in a viable condition under natural circumstances, after the loss of the embryo.

5 The amniotic cells that are taken from the embryo-clone early in its life are necessary for the case that no amniotic cells, that are originating from the amniotic fluid, have been preserved viable after birth, that is in the case that a human loses the opportunity offered by the present invention, in its first object as this is described above.

10 The specific object of the present invention wishes to solve this new technical problem. That is, each individual is given the opportunity to acquire his/her own amniotic cells, that are genetically identical to him/her and are identical to the amniotic cells that he had lost at birth, because his amniotic cells that came from amniotic fluid had not been stored after birth.

15 The amniotic cells that exist free inside the internal part of the early embryo-clone, which can be produced as above for each individual, can be taken easily by using, for example, a micropipete injection under a reverse microscope used for microinjection.

20 After being collected in the above or in another way, the clone's amniotic cells can then be multiplied in cells culture media and then cryopreserved as mentioned earlier for the amniotic cells coming from the amniotic fluid.

25 The process of embryo-clone production should not appear strange to us since already nowadays a huge number of embryos are produced, stored and then abandoned for the needs of artificial insemination.

The present object of the invention, that is the cryopreservation of amniotic cells coming from the embryo-clone at its early stages, has the same applications as the first object of the invention, that is as the
30 cryopreserved amniotic cells that come from the amniotic fluid and are taken during pregnancy.

With its further object, the present invention is offering to living persons the advantage offered to embryos according to its first object, that is the

possibility to preserve their amniotic cells so that these be useful after birth.

Therefore, the present invention offers to all living human beings, to those that are in embryonic stage as well as to those that will be born in the future, the possibility to use their own amniotic cells and to use all the benefits and advantages which genetics, biotechnology and medicine can offer based on their cryopreserved amniotic cells.

A further object of the present invention, is also every biological material which will be produced directly or indirectly from the cryopreserved amniotic cells described above; further object of the present invention is also any biological product or by-product which will be the outcome of reproduction, multiplication or extraction under the same or a modified form by the use of the above described cryopreserved amniotic cells. The said biological material, biological product or by-product will possess the same genetic properties and applications as the cryopreserved amniotic cells described above.

CLAIMS

1. Amniotic cells that are taken from amniotic fluid which surrounds the human embryo at the early stages of its development and that are
5 preserved in a viable and useful state out of their natural environment, after birth and after their natural destruction, by way of their deep-freezing (cryopreservation). These amniotic cells may be :
- a) multiplied before their cryopreservation, or
 - b) cryopreserved without previously having been multiplied, or
 - 10 c) multiplied after thawing following their cryopreservation and are again cryopreserved for long preservation.
2. Amniotic cells that are taken from human body cells, through the creation from those of embryo – clone, younger or older of the age of 14
15 days. These amniotic cells are isolated from their natural environment and are preserved, through their cryopreservation, in a viable – useful state after the end of the life of the embryo – clone. These amniotic cells may be:
- d) multiplied before their cryopreservation, or
 - 20 e) cryopreserved without previously having been multiplied, or
 - f) multiplied after thawing following their cryopreservation and are again cryopreserved for long preservation.
3. Compositions which contain a) a number of amniotic cells
25 according to each of claims 1 and 2 in different concentrations, starting from pure amniotic cells up to pure amniotic fluid, as well as b) compounds which make the amniotic cells able for long cryopreservation, such compounds being glycerin, or dimethylsulfoxide (DMSO), or polyethylene glycols (PEG's).
- 30
4. Amniotic cells according to each of claims 1, 2 and 3, which at a point in time posterior to their natural loss and destruction are used at any time :

- a) for genetic, diagnostic and therapeutic reasons.
- b) for the application on them of genetic identification.
- c) for establishing any kind of genetic identities data (such as DNA fingerprint) for diagnostic, therapeutic, social, legal, succession,
5 criminological and other purposes.

5. Amniotic cells according to claim 4, that offer a collective ready sample of genetic material and offer the possibility to identify unique genetic properties and their use and exploitation for research, diagnostic,
10 therapeutic, genetic and commercial purposes.

6. Amniotic cells according to claims 1 to 5 that are used for:
- a) on-time diagnosis and therapy of genetic diseases or genetic predisposition to diseases or functional failures,
 - 15 b) gene therapy, or for their differentiation in cells, tissues and organs for substituting the ones which suffer failures,
 - c) therapy from diseases or accidents, for the creation of cell lines resistant to pathogens, viruses, bacteria, for the creation of new cell lines, for the healing of wounds, burns, for the addition of tissues for
20 therapeutic or cosmetic purposes.

7. Amniotic cells according to claims 1, 2, 4 and 6 that are useful for the creation of differentiated cell lines categories, from the approximately two hundred (200) existing ones in the human body.

25

8. Amniotic cells according to claims 1, 2, 6 and 7, that are used through cultures to produce tissues and in the future to produce organs or even genetic clones of their owner.

30 9. Biological compound, which will be directly or indirectly produced from amniotic cells according to claims 1, 2, 5, 6, 7 and 8, as well as any biological product or by-product which will be produced from the use of such cryopreserved amniotic cells through their reproduction or

multiplication, under the same or under different form, and which possesses the same genetic properties and applications as them.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GR 00/00028

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N5/08 A01N1/02 A61K35/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS, MEDLINE, LIFESCIENCES, CHEM ABS Data, EMBASE, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PENTZ S & HÖRLER H: "A cryopreservative procedure for storing cultivated and uncultivated amniotic fluid cells in liquid nitrogen" JOURNAL OF MEDICAL GENETICS, vol. 17, 1980, pages 472-475, XP000971555 cited in the application the whole document	1-8
X	NIERMEIJER M F ET AL: "Transport and storage of amniotic fluid samples for prenatal diagnosis of metabolic diseases." HUMANGENETIK, vol. 20, no. 2, 1973, pages 175-178, XP000971556 the whole document	1-8



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

° Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

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Name and mailing address of the ISA

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Teyssier, B

INTERNATIONAL SEARCH REPORT

Intel onal Application No

PCT/GR 00/00028

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCAGGIANTE B ET AL: "Impianto di cellule epiteliali amniotiche umane crioconservate in un soggetto affetto da malattia di Niemann-Pick tipo B 'Graft of cryopreserved human amniotic epithelial cells in a subject with type B Niemann-Pick disease! " PEDIATRIA MEDICA E CHIRURGICA, vol. 9, no. 1, January 1987 (1987-01) - February 1987 (1987-02), pages 89-92, XP000971552 page 90	1-8
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E	WO 00 73421 A (PRESTIDGE PETER D ;WIGGINTON GORDON (GB); GOLDSTEIN ALLAN L (US);) 7 December 2000 (2000-12-07) page 3 -page 6	1-8

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 9

Present claim 9 relates to a compound defined by reference to its origin, namely human amniotic cells. The claim covers all compounds which can be extracted or produced from such source, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for no specific compound. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, no search was performed for claim 9.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/GR 00/00028

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